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Detection of Giardia

Technical Field

The present invention is directed to the detection of parasite pathogens, particularly *Giardia lamblia*, using molecular probes.

Background Art

Fluorescent *in situ* hybridisation (FISH) employing nucleic acid probes is one of the most advanced techniques for detection and enumeration of microorganisms. The technique emerged in the early nineties and since then has been improved rapidly and is being used for a wide range of applications which include diagnostics in clinical microbiology and analysis of microbial community structure in environmental and industrial microbiology/biotechnology. Although widely used for bacteria, very few publications describe methods for detection and enumeration of protozoan pathogens. The design of oligonucleotide probes requires skill and experience to determine accessible regions of rRNA in native ribosomes. An additional problem of successful FISH for protozoa is the development of hybridisation protocols that allow oligonucleotide probes to penetrate protozoa cell walls which are fundamentally different to bacterial cell walls. Moreover, the composition of bacterial cell walls has been well documented whereas little knowledge exists about the structure of the cyst walls of protozoa like *Cryptosporidium* spp, *Giardia* spp and related organisms.

To date, monoclonal antibodies (mabs) are the most important and widely applied tool for detection of *Giardia* cysts in water samples. The vast majority of commercially available antibodies show a lack of specificity as the antibodies detect all *Giardia* spp including species that do not infect humans. As a positive antibody reaction does not allow any conclusion regarding the viability (infectivity) of the cysts, viability stains (DAPI, PI) have to be used in conjunction with antibodies.

Oligonucleotide probes for FISH have several advantages over mabs in that probes are significantly cheaper to produce and are more stable as probes can be stored for long periods without loosing reactivity or specificity. Furthermore, correctly designed probes should only detect cysts of *Giardia lamblia* and no other species unable to infect humans. Probes target rRNA and will potentially only detect viable cysts which are able to cause infection. As non-viable (dead) cyst contain no or only small amounts of rRNA, it is envisaged that these cysts will not undergo detection.

The present inventors have developed specific oligonucleotides suitable for detection of potentially viable *Giardia* spp cysts and hybridisation protocols that allow permeabilization of the cyst walls and enable oligonucleotide probes to reach their ribosomal nucleic acid targets.

Disclosure of Invention

In a first aspect. the present invention consists in an oligonucleotide molecule for the detection of *Giardia lamblia* (*G. lamblia*). the oligonucleotide molecule hybridises to unique 18S rDNA/rRNA sequences of *G. lamblia*.

Preferably. the oligonucleotide molecule hybridises specifically to unique 18S rDNA/rRNA sequences of *G. lamblia* under medium to high stringency conditions (Sambrook *et al*.. 1989 Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press). In many cases. however. conditions of high stringency can be used to ensure specific hybridisation to unique *G. lamblia* 18S rDNA/rRNA sequences.

In a preferred embodiment of the first aspect of the present invention. the oligonucleotide molecule is selected from the group of oligonucleotides having one or more of the following nucleotide sequences:

Giar-1	GCG TCC CGG GTG AGC GGG (SEQ ID NO: 1)
Giar-2	GCC CGC GGG CGC CCG CCC (SEQ ID NO: 2)
Giar-3	TGG GCC CGC CTC GCT CGC (SEQ ID NO: 3)
Giar-4	CGG CGG GGG GCC AAC TAC (SEQ ID NO: 4)
Giar-5	GCG GGT CCA ACG GGC CTG (SEQ ID NO: 5)
Giar-6	CGG GGC TGC CGC GGC GCG (SEQ ID NO: 6)

or comprising a part of the sequences. typically at least 10 bases in length. Giar-1 to Giar-6 above so as to allow specific hybridisation to unique 18S rRNA sequences of *G. lamblia*.

In a further preferred embodiment. the oligonucleotide molecules are Giar-4 or Giar-6.

Preferably. the oligonucleotide molecules according to the invention are detectably labelled so that the oligonucleotides may be utilised as probes in hybridisation assays. It will be appreciated. however. that oligonucleotide molecules which are not labelled may be used. for example. in a polymerase chain reaction (PCR) to amplify a part of the rDNA of *G. lamblia*.

Stringent conditions are usually defined as those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% NaDodSO$_4$ at 65^{0}C; (2) employ during hybridisation a denaturing agent such as formamide, for example, up to 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 nM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42^{0}C; or (3) employ up to 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42^{0}C in 0.2 x SSC and 0.1% SDS.

Clarification of the term "18S rDNA/rRNA" for *Giardia lamblia* and *Giardia* spp. is provided as follows. The RNA molecule in question is very unusual. With approximately 1450 nucleotides, the 18S RNA molecule is significantly shorter than the 18S rRNA of other eukaryotes and in respect of sizes resembles the 16S rRNA of bacteria (Sogin et al. 1989 Phylogenetic meaning of the kingdom concept: An unusual ribosomal RNA from *Giardia lamblia*. Science **243**: 75-77). However, as determined by sequence homology *Giardia* appears to be an eukaryote representing a phylogenetically 'ancient' group of species. In the following, the term "18S rRNA/DNA" will be used for all eukaryotic sequences that were examined for the purpose of designing *Giardia lamblia* specific probes.

In a second aspect, the present invention provides a method for the detection of the presence of viable cells of *G. lamblia* in a sample, the method comprising the steps of:

(a) adding to the sample a probe comprising a detectably labelled oligonucleotide molecule according to the first aspect of the present invention;

(b) allowing hybridisation of the probe to the 18S rDNA/rRNA of any *G. lamblia* cells present in the sample; and

(c) detecting hybridisation of the probe.

Detection of any hybridisation of the probe to 18S rDNA/rRNA in the sample is indicative of the presence of viable cells of *G. lamblia* in the sample.

The sample can be any sample where there is concern that *G. lamblia* may be present. Samples include environmental, water sources, waste

materials, medical and body fluids. Examination of drinking water samples are particularly applicable for the present invention.

In a preferred embodiment, the method is used in combination with fluorescence *in situ* hybridisation (FISH) in which the oligonucleotide probe is labelled with a fluorochrome and after hybridisation, the resulting fluorescent cell is detected by epifluorescence microscopy or flow cytometry.

Suitable fluorochromes for the probes include but not limited to fluorescein isothiocyanate (FITC, green), cyanine dyes Cy2, Cy3, Cy3.5, Cy5, Cy5.5 (ranging from green to far red) or Texas Red. Other labels include radio-isotopes phosphorus ^{32}P and ^{33}P and sulfur ^{35}S. Another option is conjugation of probes to biotin and then add streptavidin-linked horseradish peroxidase (HRP) to the hybridisation reaction in order to enhance the signal via tyramide signal amplification (TSA).

In order to improve the hybridisation of the probe to the nucleic acid of the cell, the present inventors have found that adding formamide (preferably around 20% v/v) to the hybridisation buffer increases the stringency sufficiently to eliminate cross reactions of the probes with *Giardia muris*). Other agents which act in a similar manner would also be suitable to assist in specific hybridisation.

In a further preferred embodiment of the second aspect of the present invention, several different oligonucleotide probes are used and are distinguished by the use of different labels on each probe. More preferably the oligonucleotide probes are labelled with different fluorochromes and detected by flow cytometry.

While it is preferred that the probes are fluorescently labelled, it is to be understood that other known forms of labelling may be used within the broad scope of the present invention. Examples of other forms of labelling are radioactivity and chemiluminescence.

In a third aspect, the present invention provides an oligonucleotide molecule which hybridizes to *G. lamblia* 18S rDNA/rRNA sequences under medium to high stringency conditions wherein the oligonucleotide molecules hybridizes to at least one of target regions of *G. lamblia* rDNA having the following nucleotide sequences:

CCC GCT CAC CCG GGA CGC (SEQ ID NO: 7)

GGG CGG GCG CCC GCG GGC (SEQ ID NO: 8)

GCG AGC GAG GCG GGC CCA (SEQ ID NO: 9)

GTA GTT GGC CCC CCG CCG (SEQ ID NO: 10)
CAG GCC CGT TGG ACC CGC (SEQ ID NO: 11)
CGC GCC GCG GCA GCC CCG (SEQ ID NO: 12).

The oligonucleotide molecules and methods of the present invention may be used to detect the presence in a sample of any type of viable cell of *G. lamblia*. Normally only oocysts will be found in environmental samples. Other cell types, trophozoites may, however, be found and detected in clinical samples.

The oligonucleotide probes according to the present invention have tested successfully in the inventors' laboratories. Probes were used on samples that underwent IMS, staining with fluorescently labelled antibodies and sorting of positive particles on a membrane via flow cytometry. FISH was then carried out with these membranes in order to determine species identity and viability of the cysts and the membranes examined by epi-fluorescence microscopy once the hybridisation reaction is completed.

Further, it has been established that the probes specifically detect cysts of *Giardia lamblia*. Weak cross reactions were observed when the probes were hybridised against cysts of the closely related species *Giardia muris*. Cross reactions were subsequently eliminated by modifying the hybridisation buffer in a manner that increases the stringency of the hybridisation.

The present inventors demonstrated earlier that an excellent correlation exists between the FISH signal intensity obtained from *Cryptosporidium parvum* oocysts and viability of the oocysts as measured by excystation (Vesey *et al.* 1995 The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labelling of viable *Cryptosporidium parvum* oocysts. *J. Appl. Microbiol.* 85: 429-440). It is likely that *Giardia* cysts having lost their viability through ageing, which includes degradation of the rRNAs, the target of the oligonucleotide probes, will not show any fluorescent signal.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any description of prior art documents herein is not an admission that the documents form part of the common general knowledge of the relevant art in Australia.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples.

Modes for Carrying Out the Invention

Design of Oligonucleotide Probes

A brief explanation of the systematics of the genus *Giardia* is provided as follows. *Giardia lamblia* is the only species of the genus that is known to cause disease in humans. Some controversy still surrounds the systematics of the species which is also referred to as *Giardia duodenalis* or *Giardia intestinalis* (Lu *et al.* 1998 Molecular comparison of *Giardia lamblia* isolates. Int. J. Parasitol. **28**: 1341-1345). Other representatives of the genus *Giardia* described to date are *Giardia agilis* from amphibians and *Giardia muris* from rodents. birds and reptiles (Meyer 1994 *Giardia* as an organism. P 3-13. In: RCA. Thompson. J.A. Reynoldsen. A.J. Lymbery (eds.) *Giardia*: From molecules to disease. CAB International. Wallingford. Oxon. UK). *Giardia ardea* from herons (Erlandsen *et al.* 1990 Axenic culture and characterization of *Giardia ardea* from the great blue heron (*Ardea herodias*). J. Prasitol. **76**: 717-724) and *Giardia microti* from muskrats and voles (van Keulen *et al.* 1998 The sequence of *Giardia* small subunit rRNA shows that voles and muskrats are parasitized by a unique species *Giardia microti*. J. Parasitol. **84**: 294-300).

Sequence information of 18S rDNA of *Giardia lamblia* and phylogenetically closely related species was obtained from GenBank through ANGIS (Australian National Genomic Information Service) at Sydney University. All relevant sequences of *Giardia* spp. as available in April 2000 were examined. Sequences retrieved included:

Z17210-*Giardia ardea*
M54878-*Giardia lamblia*:
U09492-Giardia lamblia
U09491-*Giardia lamblia*
AF006677-*Giardia microti*
AF006676-*Giardia microti*
X65063-*Giardia muris*
U20351-*Giardia*. sp.
L16997-*Cryptosporidium parvum*:

L19069-*Cryptosporidium muris*:
L19068-*Cryptosporidium baileyi*:
U40261-*Cyclospora* sp.:
U40262-*Eimeria mitis*:
U40264-*Eimeria tenella*:
U40263-*Eimeria nieschulzi*:
U26532-*Nosema furnucalis*:
U26533-*Nosema ceranae*:
X73894-*Nosema apis*: and
L39110-*Ichtyosporidium*

Sequences were aligned using the program ClustalW and screened for diagnostic regions. e.g. regions that specifically discriminated *Giardia lamblia* from the other species included in the alignment. Six target regions were identified as listed below. The positions of the nucleotides given are not based on any internationally recognised numbering system but refer to the numbering for the *Giardia lamblia* 18S rRNA (designated 16S rRNA in the reference) given in a secondary structure model published by Sogin *et al.* in 1989 (Phylogenetic meaning of the kingdom concept: An unusual ribosomal RNA from *Giardia lamblia*. Science **243:** 75-77). In accord with international agreements, all sequences listed, including oligonucleotide sequences, are shown in 5'-3' orientation.

Target Regions

Target regions (rDNA) were identified as follows:

1. CCC GCT CAC CCG GGA CGC (Position 57-74) (SEQ ID NO: 7)
2. GGG CGG GCG CCC GCG GGC (Position 166-183) (SEQ ID NO: 8)
3. GCG AGC GAG GCG GGC CCA (Position 391-408) (SEQ ID NO: 9)
4. GTA GTT GGC CCC CCG CCG (Position 508-525) (SEQ ID NO: 10)
5. CAG GCC CGT TGG ACC CGC (Position 552-569) (SEQ ID NO: 11)
6. CGC GCC GCG GCA GCC CCG (Position 596-613) (SEQ ID NO: 12)

Due to the fact that *G. lamblia* shows a very unusual 18 rRNA regarding sequence and secondary structure, it appeared reasonable to assume that the probes are *G. lamblia* specific and will not show cross reactions with other protozoans under moderately stringent hybridisation conditions. Table 1 shows a comparison of the target regions for the two functional FISH probes

Giar-4 and Giar-6 from all *Giardia* spp. 18S rDNA/RNA target sequences available. A cross reaction observed from probe Giar-4 with *G. muris*, probably the phylogenetically closest related species to *G. lamblia*, occurred under low stringency hybridisation and was eliminated by increased stringency through 20 % formamide in the hybridisation buffer. The alignment of published sequences used to design the probes showed that the corresponding target region of Giar-4 on the *G. muris* 18S rRNA shows eight mismatches and one deletion compared to the target region on the *G. lamblia* 18S rRNA. A corresponding target region for Giar-6 does not exist on the *G. muris* 18S rRNA. It appears that a large part of the rRNA in this region was deleted during the evolution of *G. muris*.

From database searches, it would appear that the Giar-4 and Giar-6 probes might cross react with a species designated *G. microti*, isolated from muskrats and voles. These sequences show a very high overall sequence homology up to 96.8 % to the *G. lamblia* sequence. It appears questionable to describe the isolate as a species different to *G. lamblia* as other *Giardia* spp. share as little as 72-75 % sequence homology *G. lamblia*. Given that it has not been demonstrated to date that *G. microti* does not infect humans, it appears possible that the organism is in fact *G. lamblia* or a subspecies of *G. lamblia* which is supported by the high sequence homology of the 18S rRNAs. According to literature available, *G. microti* is not a generally recognised species and the fact that Giar-4 and Giar-6 will detect the organism is unlikely to contradict the finding that the probes according to the present invention are *G. lamblia* specific.

TABLE 1: Comparison of the target regions of 'Giar-4' and 'Giar-6' on the 16S rDNA of *Giardia* spp.*

Species/ Accession	Target 'Giar-4'	Target 'Giar-6'
G. lamblia M54878	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. lamblia U09492	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. lamblia U09491	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. microti AF006677	GTAG TTGGCCCCCCGCCG	CTCGC CGCGGCA GCCCCG
G. microti AF006676	GTAG TTGGCCCCCCGCCG	CGCGC CGCGGCA GCCCCG
G. ardea Z17210	GCAGGCGTCGCGCGGCGCTG	TGGACCTACCGCCCGGGACGGCG
G. sp. U20351	GGCGCTGCTG CTGCAGTTA	CGC C CGGGAC GCGCG
G. muris X65063	GGAGTCGAGACGTC CAG	Not Applicable**

Nucleotide residues printed in bold indicate mismatch to the *G. lamblia* target sequences of 'Giar-4' and 'Giar-6'. blank space represents nucleotide deletions

*Analysis include all 16S rDNA sequences of *Giardia* spp. available through GenBank in April 2000

**Comparative sequence analysis and secondary structure modeling led to the conclusion that no corresponding target region exists on the 16S rRNA of *G. muris*

FISH Probes

Fluorescently labelled oligonucleotide probes were produced for the target regions as shown above. Naturally, sequences of the oligonucleotides are the reverse complement of the target regions:

Giar-1	GCG TCC CGG GTG AGC GGG	(SEQ ID NO: 1)
Giar-2	GCC CGC GGG CGC CCG CCC	(SEQ ID NO: 2)
Giar-3	TGG GCC CGC CTC GCT CGC	(SEQ ID NO: 3)
Giar-4	CGG CGG GGG GCC AAC TAC	(SEQ ID NO: 4)
Giar-5	GCG GGT CCA ACG GGC CTG	(SEQ ID NO: 5)
Giar-6	CGG GGC TGC CGC GGC GCG	(SEQ ID NO: 6)

Preliminary investigations including a universal eukaryotic probe as a positive control showed that only probes Giar-4 and Giar-6 were suitable for FISH as the other probes yielded no or very weak signals. Subsequently, Giar-4 and Giar-6 were employed for further testing and refinement of hybridisation conditions. The other four probes, Giar-1, Giar-2, Giar-3, and Giar-5, however, can be employed for specific detection of *Giardia lamblia* rRNA or rDNA in techniques that target free nucleic acids such as Polymerase Chain Reaction (PCR) assays or dot blot hybridisations.

RESULTS

Commercially available viable *Giardia lamblia* cysts were used. Aliquots of cysts were stored at -20°C in 50% ethanol and 50% phosphate buffered saline (PBS), pH 7.2. This method of fixation enables long term storage (> 1 year) prior to hybridisation experiments without diminished FISH signal due to degradation of ribosomal nucleic acids.

METHOD

Protocol for Fluorescent *in situ* Hybridisation in 1.5 ml Tubes

A suspension containing cysts was centrifuged for 5 min and the supernatant discarded. A centrifugal force greater than 1200 x g should be avoided as this will cause many cysts to rupture.

Resuspend cysts in 50% ethanol and 50% PBS, incubate at 80°C for 20 min.

Centrifuge cysts, discard supernatant and resuspend in FISH buffer (0.9 M NaCl, 10 mM Tris/HCl, pH 7.2, 0.1% SDS, 20% formamide). Adding 20% formamide to the hybridisation buffer increased the stringency

sufficiently to eliminate the above mentioned cross reactions of the probes with *Giardia muris* containing 1 picomol per microliter of each fluorescently labelled probe Giar-4 and Giar-6.

Incubate at 80°C for 2 min. transfer to 48°C water bath and incubate 60 min.

Terminate hybridisation by adding ice cold PBS. spin. discard supernatant and resuspend cysts in ice cold PBS. Cysts are then ready to be examined.

The hybridisation protocol as detailed above can be applied for cysts concentrated via IMS. stained with FITC-labelled antibodies and sorted on filters by flow cytometry. Alternatively. *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts can be detected simultaneously by applying the specific probes described here in conjunction with *Cryptosporidium parvum* specific probes and hybridisation protocols (Deere *et al.* 1998 Rapid method for fluorescent *in situ* ribosomal RNA labelling of *Cryptosporidium parvum*. *J. Appl. Microbiol.* 85: 807-818: Vesey *et al.* 1998 The use of a ribosomal RNA targeted oligonucleotide probe for fluorescent labelling of viable *Cryptosporidium parvum* oocysts. *J. Appl. Microbiol.* 85: 429-440).

Modifications

Possible modifications of the invention is the application of methods that allow amplification of the fluorescent FISH signal. In brief. molecular beacons are modified. fluorescently labelled oligonucleotides that can only emit a fluorescent signal when attached to their specific nucleic acid target. No signal will be obtained from probes that are bound non-specifically to any other matter in a sample. In essence. increased signal strength is achieved by eliminating or at least drastically reducing background signals (Schofield *et al.* 1997 Molecular beacons: Trial of a fluorescence-based solution hybridization technique for ecological studies with ruminal bacteria. *Appl. Envir. Microbiol.* 63: 1143-1147). Tyramide signal amplification (Schönhuber *et al.* 1997 Improved sensitivity of whole cell hybridization by the combination of horseradish peroxidase-labelled oligonucleotides and tyramide signal amplification. *Appl. Envir. Microbiol.* 63: 3268-3273) utilises unlabelled oligonucleotide probes that are conjugated to horseradish peroxidase (HRP). After the hybridisation reaction is completed, the hybridisation buffer is removed and replaced by a buffer solution containing HRP substrate conjugated to a fluorescent dye. Subsequently. HRP linked to

oligonucleotides converts the substrate into a fluorescent precipitate that accumulates in the target cells.

The present invention is a continuation of the development of specific oligonucleotide probes for detection and enumeration of protozoan pathogens in water via fluorescent *in situ* hybridisation (FISH). The investigations led to the first specific FISH probes for detection of viable *Cryptosporidium parvum* oocysts (WO 96/34978 entitled Method for the Detection of Viable *Cryptosporidium parvum* oocysts). Comparative sequence analysis of the 18S ribosomal DNA (rDNA) from *Giardia lamblia* and closely related species (in terms of sequence homology) and secondary structure analysis was used to determine the accessibility of the potential target region resulted in the oligonucleotide probes disclosed. Based on the above investigations, six potentially specific FISH probe sequences were determined. The probes were produced and tested on trophozoites of *Giardia lamblia*. Two of these probes (designated Giar-4 and Giar-6) yielded a strong fluorescent signal and showed the same result when hybridised against potentially viable cysts. The remaining four probes showed either very weak or no fluorescence at all and were subsequently omitted from further FISH experiments. However, these four probes can be employed for specific detection of *Giardia lamblia* rRNA or rDNA in techniques that target free nucleic acids such as Polymerase Chain Reaction (PCR) assays or dot blot hybridisations.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.